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Distance-dependent translational coupling and interference in *Lactococcus lactis*

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Summary. The possibility of raising the expression level of a heterologous gene in *Lactococcus lactis* by exploiting the principle of translational coupling was investigated. For this purpose, the *Escherichia coli lacZ* gene was transcriptionally fused to a short open reading frame (ORF) of lactococcal origin. A Shine-Dalgarno (SD) sequence was introduced at the boundary of the two ORFs. In a series of otherwise identical plasmids, the relative positions of the translational stop codon of the upstream ORF and the translational start codon of the downstream ORF (*lacZ*) were varied. The expression of *lacZ* gradually increased as the stop and start codons were placed in closer proximity. A concomitant switch from translational interference to translational coupling was observed. Best results were obtained with partially overlapping stop and start codons. It is concluded that the principle of translational coupling offers good possibilities to increase the level of heterologous gene expression in *L. lactis*.

Key words: *Lactococcus lactis* – Heterologous gene expression – Translational coupling – Translational interference

Introduction

Recent years have seen an increased interest in the genetics of lactic acid bacteria. Methods have been developed to make these organisms accessible to genetic modification techniques (for a review, see De Vos 1987). This offers the possibility of adjusting the properties of these bacteria to make them more suitable for use in dairy, meat and vegetable fermentations. An obviously important way to broaden the applicability of lactic acid bacteria is to provide them with new traits by the incorporation of heterologous genes. To this end general purpose cloning vectors have been developed (Kok et al. 1984). Gene expression signals have been isolated and characterized (Van der Vossen et al. 1987; de Vos 1987), and expression vectors developed (Van de Guchte et al. 1989). Using these tools, several heterologous genes have been expressed in *Lactococcus lactis*. However, depending on the nature of newly introduced genes, high levels of expression may be desired. One way to accomplish this is to increase the gene dosage, for example by using high copy number plasmid vectors. Another possibility is to increase the efficiency of expression of the gene by improving the processes of transcription and translation. In this study we concentrated on the translation process. The DNA sequences usually regarded as being the most important determinants of translation initiation efficiency are the highly conserved Shine-Dalgarno (SD) sequence, the translational start codon, and the spacer region between the two. However, sequences within the coding region of the gene may also be important. They may exert their influence via their primary nucleotide sequence, which may contribute to the interaction between the mRNA and the 16S rRNA (Hager and Rabinowitz 1985; Sprengart et al. 1990), or via a contribution to the formation of secondary mRNA structures in the translation initiation region (De Smit and Van Duin 1990). When the coding region of a gene is replaced with that of another gene, it remains uncertain how efficient initiation of translation of the latter gene will be. Even if it were possible to predict a negative effect on efficiency, it will often be difficult to adjust the coding region in such a way that the effect is abolished without affecting the amino acid sequence of the gene product. Therefore, it may be advantageous to use a homologous translation initiation region, including a short open reading frame (ORF), to express a heterologous gene by means of translational coupling in a two-cistron system. The aim of this work was to investigate whether this approach could be used for the improvement of heterologous gene expression in *L. lactis*.

The principle of translational coupling, first described by Oppenheim and Yanofsky (1980), ensures the efficient translation of a gene by making it dependent on the

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translation of a gene immediately preceding it. Several naturally occurring as well as artificial systems of translational coupling have been reported, that have proved more or less successful in improving gene expression. In these systems, the relative positions of the stop codon of the first ORF and the start codon of the second ORF vary (Das and Yanofsky 1984; Schümperli et al. 1982; Harms et al. 1988; Little et al. 1989; Schoner et al. 1984; Oppenheim and Yanofsky 1980; Gatenby et al. 1989; Lindahl et al. 1989). Since many additional differences existed in the systems reported, e.g. the nature of the ORFs and the ribosome binding sites (RBSs) involved, the optimal configuration of the translational stop and start codons in translational coupling remained largely undetermined. In this report we present the results of a systematic study on translational coupling, involving a series of plasmids in which the only variable was the distance between the stop codon of one ORF and the start codon of the downstream ORF.

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant feature(s)	Source or reference
Bacteria		
Escherichia coli		
MC1000	*araD*139, *lacX*74 (*ara, leu*)7697 *galU, galK, strA*	Casadaban and Cohen (1980)
Lactococcus lactis subsp. *lactis*		
IL1403	Plasmid free	Chopin et al. (1984)
Plasmids		
pBR322	Amp^r, Tc^r	Bolivar et al. (1977)
pMLB1034	Amp^r; carrying a 5′-truncated *lacZ* gene	Casadaban et al. (1980)
pMG36e	Em^r; lactococcal expression vector	Van de Guchte et al. (1989)
pMG36eHEL	Em^r; pMG36e derivative carrying the mature hen egg white lysozyme cDNA sequence	Van de Guchte et al. (1989)
pMG36HEL	Km^r; otherwise as pMG36eHEL	Van de Guchte et al. (1989)
pMG57	Em^r; *lacZ* fusion vector	This work
pMG59	Em^r; in-frame fusion of ORF32 and *lacZ*	This work
pMG60	Em^r; pMG59 derivative lacking ORF32	This work
pMG53	Em^r; as pMG60, with changed ribosome binding site	This work
pTC1–pTC6	Em^r; constructs used to study translational coupling	This work
pTU1–pTU6	Em^r; pTC1–pTC6 derivatives in which ORF32 translation is prevented	This work

Materials and methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids containing *lacZ* gene fusions are all based on pMG57 (Fig. 1). This plasmid is composed of a *Hin*dIII-*Sma*I fragment carrying the replication and gene expression functions of the lactococcal expression vector pMG36e (Van de Guchte et al. 1989), and a *Sma*I-*Dra*I fragment containing a 5′-truncated *Escherichia coli lacZ* gene derived from pMLB1034 (Casadaban et al. 1980). The *Bam*HI site originally present in the first part of *lacZ* in pMLB1034 had been removed by mung bean nuclease treatment, followed by religation. The *Sma*I-generated ends of both fragments were ligated to give a new *Sma*I site, whereas the *Hin*dIII- and *Dra*I-generated ends were linked via a five nucleotide pBR322-derived *Hin*dIII-*Cla*I sequence, the *Cla*I site of which had been filled in with Klenow enzyme. Plasmid pMG57 contains the broad host range origin of replication originally derived from pWV01 (Kok et al. 1984), which enables it to replicate in *E. coli, L. lactis,* and several other bacterial species. Upstream of *lacZ* a lactococcal promoter (P32) is present, followed by a short ORF (ORF32) and a unique *Sma*I restriction site. P32 and the first nine codons of ORF32 were isolated as one unit from *L. lactis* subsp. *cremoris* Wg2 chromosomal DNA (Van der Vossen et al. 1987). The *lacZ* gene in pMG57 has a reading frame different from that of ORF32 (Fig. 2).

In the *Sma*I site of pMG57 DNA sequences were inserted that were generated in either of two ways: fragments present in plasmids pTC1 to pTC3, pTC5 and pTC6 (Fig. 2) were generated by the polymerase chain reaction (PCR) using pMG36eHEL (Van de Guchte et al. 1989) as a template. In each case one of the primers used in the DNA amplification reaction was designed to introduce the desired mutations, as well as an appropriate restriction enzyme site to allow the recloning of

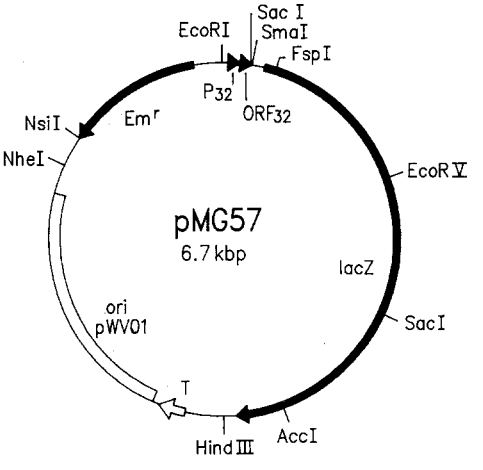

Fig. 1. Plasmid pMG57. Em^r, erythromycin resistance marker; *lacZ*, 5′-truncated *Escherichia coli lacZ* gene; P32, lactococcal promoter 32 (Van der Vossen et al. 1987); ORF32, open reading frame 32 (Van der Vossen et al. 1987); ori pWV01, replication origin of the *Lactococcas lactis* subsp. *cremoris* plasmid pWV01 (Kok et al. 1984); T, transcription terminator (Kok et al. 1988)

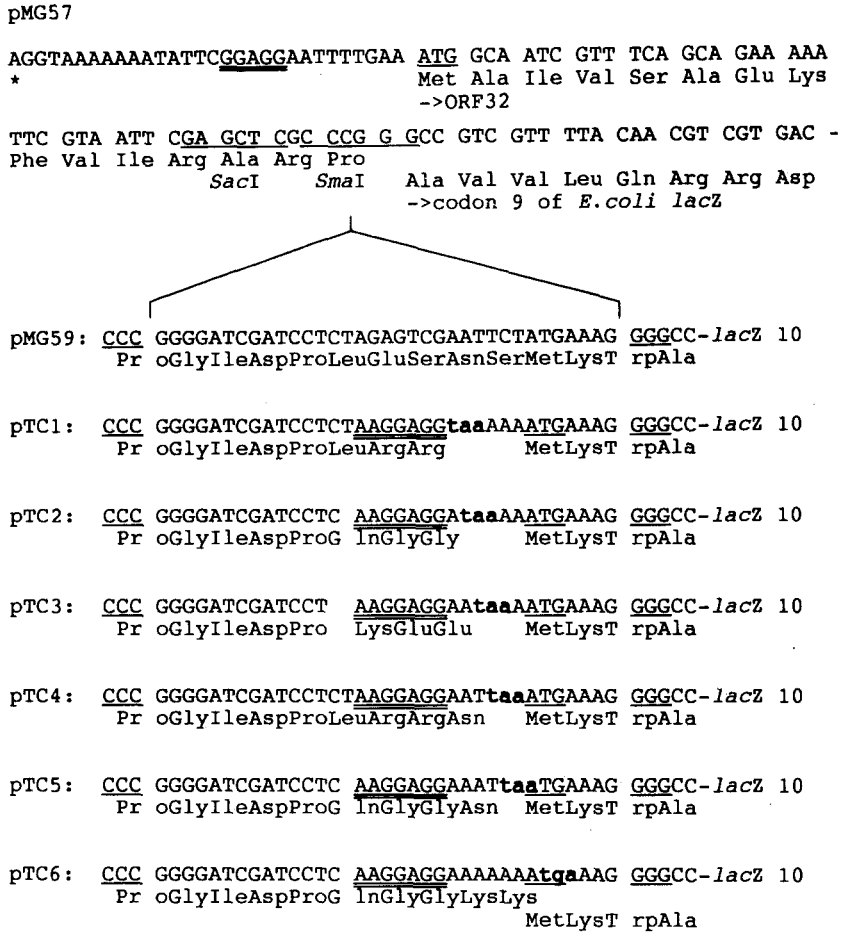

Fig. 2. DNA sequences preceding the *lacZ* gene in pMG57 and its derivatives. Shine-Dalgarno (SD) sequences are *doubly underlined.* *, transcriptional start point (Van der Vossen et al. 1987); *underlined* ATG, translational start codon; taa, tga, in *bold*, tanslational stop codons; *lac*Z 10, codon 10 of the *Escherichia coli lacZ* gene

the mutated DNA fragment upstream of the *lacZ* gene. At the opposite (5′) end the mutated fragments were bounded by the *Sma*I site present in pMG36eHEL. Fragments present in pMG59 and pTC4 were produced by site-directed mutagenesis following the gapped duplex approach in the pMac system (Stanssens et al. 1989). For this purpose, part of pMG36HEL (Van de Guchte et al. 1989) was recloned in the pMa phasmid. Again, the mutagenic primers were made such that a restriction enzyme site to be used for the recloning of the altered DNA fragment was introduced.

Plasmids pMG60 and pMG53 (Fig. 3) were produced in a similar way, with the modification that the promoter-bearing fragment (*Eco*RI-*Sma*I) of pMG57 was replaced with a nearly identical fragment lacking the ORF32 sequence. In addition, the RBS in pMG53 was changed to make it identical to the one immediately preceding the *lacZ* gene in pTC4.

Plasmids pTU1 to pTU6 were obtained by replacing the *Eco*RI-*Sac*I fragment (Fig. 1) in plasmids pTC1 to pTC6 with a nearly identical fragment in which the sequence comprising the start of ORF32, GAA*ATG*, had been changed to CCCGGG, thereby abolishing translation of ORF32 in these constructs.

pMG57 and derivatives were obtained in *E. coli* MC1000, with selection for erythromycin resistance and blue colour development on TY agar containing the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Relevant parts of the DNA sequences were checked by DNA sequencing. Subsequently, the plasmids were used to transform *L. lactis* subsp. *lactis* IL1403 by electroporation (Van der Lelie et al. 1988).

Media. E. coli was grown in TY broth (Rottlander and Trautner 1970) or on TY solidified with 1.5% agar. *L. lactis* was grown in glucose M17 broth (Terzaghi and

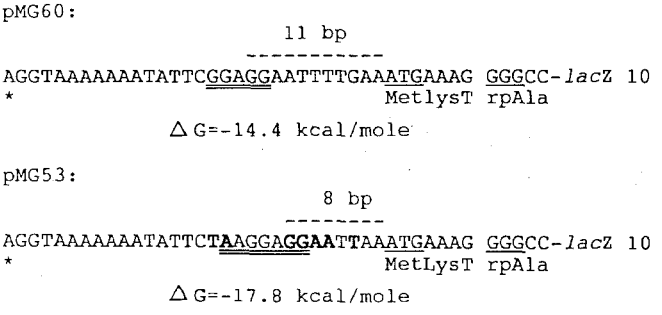

Fig. 3. The *lacZ* translation initiation regions in pMG60 and pMG53. SD sequences are *doubly underlined.* The size of the window is given above the sequence. *, transcriptional start point (Van der Vossen et al. 1987); *underlined* ATG, translational start codon; *lacZ* 10, codon 10 of the *Escherichia coli lacZ* gene; *ΔG*, free energy (Tinoco et al. 1973) of the complementarity between the SD sequence and the 3′ end of the 16S rRNA of *Lactococcus lactis* (Ludwig et al. 1985)

Sandine 1975), or on glucose M17 solidified with 1.5% agar. Sucrose (0.3 M) was added to these media to stabilize electroporated cells osmotically. Erythromycin was used at 100 μg/ml and 5 μg/ml for *E. coli* and *L. lactis*, respectively. X-gal (Sigma, St. Louis) was added to a final concentration of 40 μg/ml.

DNA manipulations. Plasmid DNA was isolated by the method of Birnboim and Doly (1979). Restriction enzymes, Klenow enzyme, T4 DNA ligase, and *Taq* DNA polymerase were purchased from Boehringer (Mannheim, FRG) and used according to the instructions of the supplier. For PCR-mediated DNA amplification a Bio-med thermocycler 60 (B. Braun, Melsungen, FRG) was used.

Sequence analysis. DNA sequence analysis was performed according to Tabor and Richardson (1987) using the T7 polymerase system (Pharmacia, Uppsala, Sweden) on denatured plasmid DNA.

Assay of β-galactosidase activity. β-galactosidase activity was determined in overnight-grown cultures. Cells from 25 ml of the cultures were collected by centrifugation and resuspended in 2 ml of cold Z-buffer (Miller 1972). To 1.2 ml of this cell suspension 1 ml of glass beads (0.1 mm in diameter) was added and the cells were disrupted using the "Shake it, Baby" cell disrupter (Biospec Products, Bartlesville, Oklahoma) (two cycles of 5 min each, at maximum speed setting, at 4° C). Cell debris was removed by centrifugation in an Eppendorf centrifuge. The protein content of the lysates was determined according to Bradford (1976), and β-galactosidase activity per milligram protein was determined essentially as described by Miller (1972).

Results

Translational control regions preceding the lacZ *gene*

A schematic overview of the structures present upstream of the *lacZ* gene in the various plasmids used is presented in Fig. 4. DNA sequence details are given in Figs. 2 and 3. In pMG59 an in-frame fusion between ORF32 and the *lacZ* gene is present. Instead of the original N-terminal 8 *lacZ* codons, the fused gene contains 27 other codons, of which the 1st and the 25th are ATGs. Of these 27 codons, the first 24 are not present in the otherwise identical plasmid pMG60. The ATG corresponding to codon 25 in pMG59 serves as the translational start codon in pMG60. pMG53 specifies the same β-galactosidase derivative as pMG60; however, the RBS has been changed: the SD sequence was altered as well as the spacing between this sequence and the ATG start codon (Fig. 3). These changes result in a lower value for the calculated free energy of binding to the 3′ end of *L. lactis* 16S rRNA. In the plasmid series pTC1 to pTC6, a two-cistron system was created based on the sequence present in pMG59. For this purpose, a translational stop codon was inserted to terminate translation of ORF32.

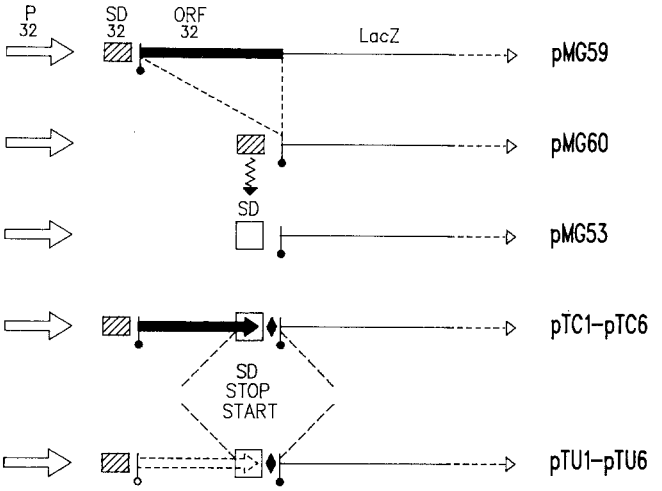

Fig. 4. Schematic overview of translation initiation regions studied. ⇨, lactococcal promoter 32; □, ▨, SD sequences; ♦, translational start codon (START); ◊, translational start codon removed; ➡, translated ORF32, ⇨, non-translated ORF32; ◆, translational stop codon (STOP); *LacZ*, 5′-truncated *Escherichia coli lacZ* gene

An SD sequence was introduced to permit the separate translation of the second ORF, that is, the modified *lacZ* gene as present in pMG60 and pMG53. The RBS formed upstream of *lacZ* is the same as that in pMG53. Plasmids pTC1 to pTC6 differ in the relative positions of the stop codon of ORF32 and the start codon of *lacZ*. As can be seen in Fig. 2, these codons are separated by 3 to 1 nucleotides in pTC1 to pTC3, are contiguous in pTC4, and partially overlap in pTC5 and pTC6. In pTC1 to pTC6 the SD sequence and the spacing between this sequence and the start codon of *lacZ* were identical. Minor differences had to be introduced in the area preceding the SD sequence to keep the stop codon in-frame with ORF32. Finally, in the plasmid series pTU1 to pTU6 translation of ORF32 was prevented by conversion of the sequence-GAA*ATG*-, comprising the translational start codon in the pTC plasmids, to -CCCGGG- in the pTU derivatives.

lacZ *gene expression in* L. lactis

The results of β-galactosidase activity determinations in *L. lactis* strains carrying the plasmids described above are presented in Fig. 5A and B. All data were related to the activity observed in strain IL1403 (pMG60) which was taken to be 100%. IL1403 (pMG57) showed no detectable β-galactosidase activity. The strain carrying pMG59 showed a β-galactosidase activity eight times higher than that of the strain carrying pMG60 (result not shown). Assuming that the two *lacZ* products specified by pMG59 and pMG60 do not differ in stability or specific activity, this result suggests that translation initiation was more efficient in pMG59 than in pMG60. Figure 5 shows that the mere alteration of the RBS in pMG53, as compared with pMG60, raised the activity level by 18%. The two-cistron systems as present in the pTC plasmids gave rise to β-galactosidase activity levels

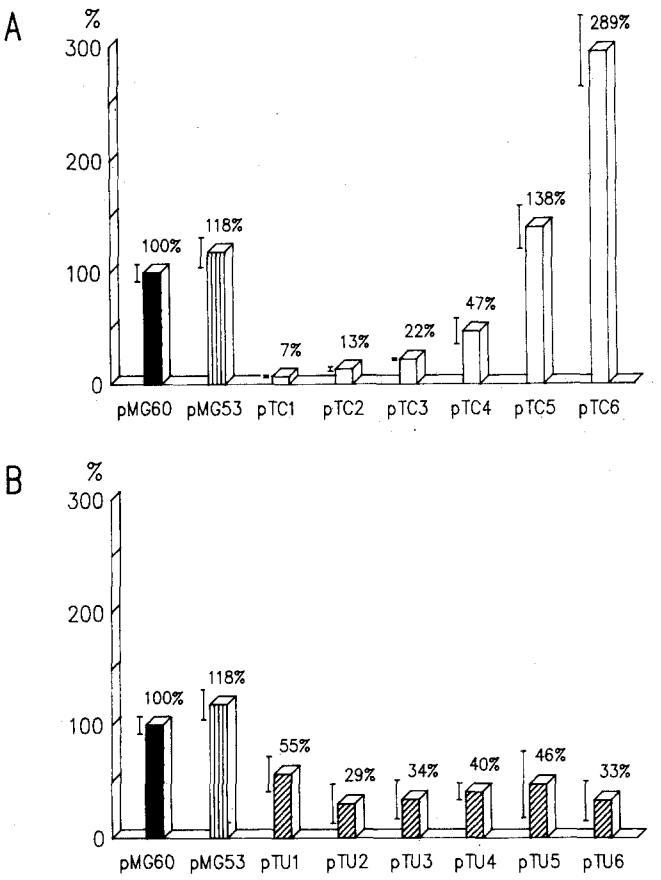

Fig. 5A and B. Relative β-galactosidase activities in *Lactococcus lactis* strains harbouring the plasmids indicated on the χ-axis. β-galctosidase activity of IL1403 (pMG60) is set to 100%. I, standard deviation

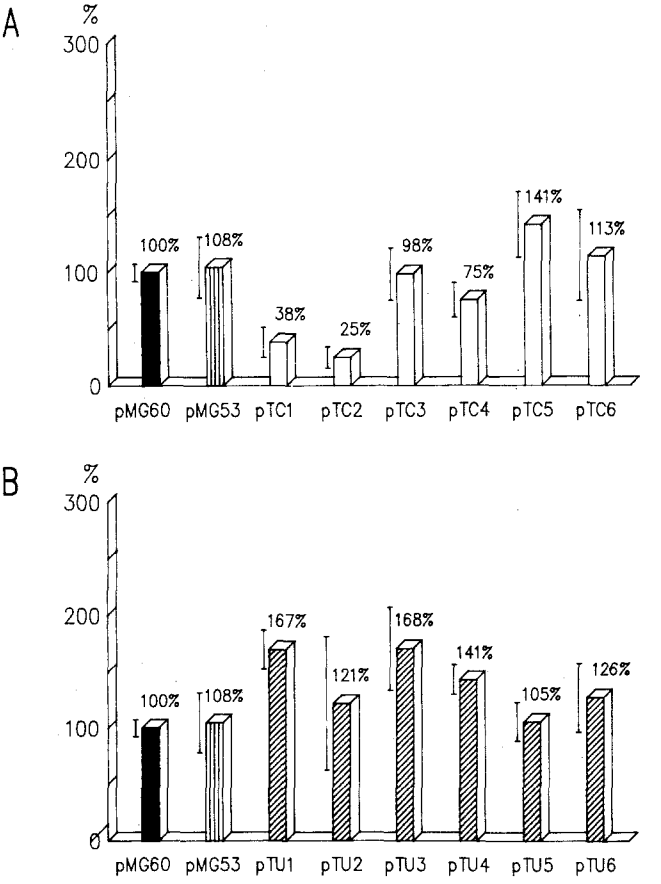

Fig. 6A and B. Relative β-galactosidase activities in *Escherichia coli* strains harbouring the plasmids indicated on the χ-axis. β-galactosidase activity of MC1000 (pMG60) is set to 100%. I, standard deviation

ranging from 7% in IL1403 (pTC1) to 289% in IL1403 (pTC6) relative to that of IL1403 (pMG60). The expression level of the *lacZ* gene gradually increased as the distance between the stop codon of ORF32 and the start codon of *lacZ* decreased. The best results were obtained with partially overlapping stop and start codons as present in pTC5 and pTC6. Only in these configurations were expression levels higher than that of IL1403 (pMG60) and IL1403 (pMG53) attained. The expression of *lacZ* in the two-cistron systems is strongly influenced by the translation of the preceding ORF, as is apparent from a comparison of the results presented in Fig. 5A and B. When translation of ORF32 was prevented, as was the case in plasmids pTU1 to pTU6, the β-galactosidase activities levelled off at about 40% of the activity reached by IL1403 (pMG60), with only small differences between the various constructs. Strains harbouring the plasmids pTC4 to pTC6 reached higher activity levels than the strains harbouring the corresponding pTU plasmids. The most marked difference can be seen in the comparison of IL1403 (pTC6) and IL1403 (pTU6), where the former strain showed an activity level about nine times higher than that of the latter. Strains carrying plasmids pTC1 to pTC3, however, showed a reduced expression of *lacZ* when compared with strains containing the plasmids pTU1 to pTU3.

lacZ *gene expression in* E. coli

The results of β-galactosidase activity measurements in *E. coli* are presented in Fig. 6A and B. The activity observed in strain MC1000 (pMG60) was set at 100%, and data obtained for the other strains were related to this value. When expressed in β-galactosidase activity units, the 100% value obtained in *E. coli* was approximately 180 times higher than that obtained in *L. lactis*. Plasmid pMG57 gave rise to detectable β-galactosidase activity in *E. coli* MC1000, amounting to 1%–2% of the value obtained with MC1000 (pMG60). In contrast to the observation made in *L. lactis*, in *E. coli* there was hardly any difference in the β-galactosidase activity values obtained for strains carrying plasmids pMG59 and pMG60 (result not shown). The difference in the RBSs of pMG53 and pMG60 did not result in significantly different β-galactosidase activity levels. Also, the effect of the relative positions of the stop and start codons in pTC1 to pTC6 was not as clear as in *L. lactis*, although, here also, the configurations with partially overlapping stop and start codons seemed to be the more favourable (Fig. 6A). Only strain MC1000 (pTC5) reached an activity level clearly higher than that of MC1000 (pMG60) and MC1000 (pMG53). Surprisingly, plasmids pTU1 to pTU6 all gave rise to β-galactosidase

activity levels equal to or exceeding those of MC1000 (pMG53) (Fig. 6B). Moreover, in all cases except MC1000 (pTU5), the expression levels reached with the pTU plasmids were higher than with the corresponding pTC plasmids.

Discussion

In the present study the possibility of using translational coupling for the improvement of heterologous gene expression in *L. lactis* was investigated. We concentrated on systems in which the stop codon in a lactococcal ORF (ORF32) and the start codon of a modified *E. coli lacZ* gene were closely linked, and performed a systematic analysis to determine the optimal configuration of the two codons. Although similar systems have been described before, the results of these studies could not be correlated because of the diversity of the various components (Das and Yanofsky 1984; Schümperli et al. 1982; Harms et al. 1988; Little et al. 1989; Schoner et al. 1984; Oppenheim and Yanofsky 1980; Gatenby et al. 1989; Lindahl et al. 1989).

In *L. lactis* a clear effect of the relative positions of the stop and start codons on the expression of *lacZ* downstream of ORF32 was observed. Reduction of the distance between the two codons resulted in a gradual increase in the production of β-galactosidase. The best results were obtained with overlapping stop and start codons. From a comparison with the results obtained with plasmids in which the translation of ORF32 was prevented, it can be concluded that in this model system translational coupling occurs with contiguous or overlapping stop and start codons. When the stop and start codons are separated by 1 to 3 bases, translational interference is observed. In these cases a higher expression level was reached when ORF32 was not translated. Only in cases in which ORF32 was translated and in which the stop and start codons were partially overlapping, was a higher expression level than that attained in the absence of ORF32 observed.

Three hypotheses have been formulated concerning the molecular events underlying the principle of translational coupling (Oppenheim and Yanofsky 1980). In one, translation of an upstream ORF is thought to resolve secondary structures in the mRNA, thereby giving ribosomes access to the otherwise occluded RBS of the downstream ORF. Although several cases of translational coupling described in the literature can be satisfactorily explained on the basis of this idea, we believe that it cannot explain the results presented here, because it is hard to imagine how differences of only a few basepairs in the separation of the two ORFs could have a substantial and systematic effect on the accessibility of the RBS to the ribosomes. The second hypothesis, which assumes that translation of the first ORF results in a high local concentration of ribosomes that can initiate translation of the second ORF, is rejected on the same basis. A third hypothesis envisages a so-called translational restart, using ribosomes that have translated the first ORF and that do not dissociate from the mRNA. Our results seem to fit this hypothesis quite well, as the efficiency of a translational restart system would critically depend on a correct juxtaposition of translational stop and start signals.

In the context of the translational restart hypothesis, an important prerequisite of which is that the initiation of translation of the upstream ORF is more efficient than that of the downstream ORF, we have to assume that the presence of ORF32 results in a more efficient initiation of translation than the presence of the modified *lacZ* gene. Assuming that there are no differences in RNA levels, this may explain the difference in the β-galactosidase activities obtained with pMG60 and pTC6 (Fig. 5A), which contain identical non-coding sequences upstream of *lacZ* and of ORF32, respectively (Fig. 3). It may partly explain the difference between IL1403 (pMG59) and IL1403 (pMG60), although in this case product stability may also be involved.

In *E. coli* no clear translational coupling effect was observed. One explanation may be that in *E. coli* the two translation initiation regions result in similar efficiencies of translation initiation, thereby eliminating the basis for enhanced gene expression by translational restart. It is not unusual that changes in translation initiation regions have an effect on translation efficiency in gram-positive bacteria (i.e. *Bacillus subtilis*) but not in *E. coli* (Hager and Rabinowitz 1985).

Although the exact molecular events underlying translational coupling remain to be established, our results conclusively show that in *L. lactis* translational coupling can contribute to an improvement in heterologous gene expression. The system may be further optimized by using lactococcal sequences other than the combination P32-ORF32. The results presented here also bear on the use of promoter probe vectors for the determination of promoter strength. A commonly used technique for the isolation and characterization of promoter-like structures is to insert randomly cut chromosomal DNA fragments upstream of a reporter gene in a pomoter probe vector, and to determine the relative promoter strength by measuring the activity of the gene product. It is clear from our results that the presence and translation of an ORF in between a promoter and the reporter gene can have important implications for the expression level of this gene. This should be kept in mind when interpreting the results of promoter strength determinations.

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